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The evolution of prezygotic reproductive isolation in the Drosophila pseudoobscura subgroup

Sheri Dixon Schully
Louisiana State University and Agricultural and Mechanical College, sdixon1@lsu.edu

THE EVOLUTION OF PREZYGOTIC REPRODUCTIVE ISOLATION IN THE *DROSOPHILA PSEUDOOBSCURA* SUBGROUP

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DEDICATION

I dedicate this dissertation to my parents, Lydia and Dale Dixon. My dad taught me the values of first-rate hard work. My mother has always made me feel that I could accomplish anything I put my mind and heart into. It has been her belief in me that has gotten me this far.

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Last but certainly not least, I would like to thank my husband Kevin Schully. He is my colleague, my support system and my best friend. My marriage to him is one of the best things that came out of all of this hard work.

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ABSTRACT

Newly forming species that have differentiated in allopatry may evolve numerous barriers that prevent the interbreeding when they come back into contact with each other. The objective of this dissertation is to evaluate some mechanisms of prezygotic reproductive isolation in the *D. pseudoobscura* subgroup. I begin by evaluating how the evolution of female preferences and male sexual characters lead to reinforcement between *Drosophila pseudoobscur*a and its congener *D. persimils*. In particular, I will evaluate two alternative hypotheses; Preference Evolution and Discrimination Enhancement, to determine how selection reduces hybridization between these sister species. Both hypotheses predict a reduction in the overlap of male traits and female preferences in hybridizing populations; however, the target of selection differs between the two. Next, I will discuss reproductive isolation as a result of competiton between gametes, in particular conspecific sperm precedence. Until this study, patterns of sperm precedence had rarely been examined between divergent populations or subspecies within a species. I will evaluate conspecific sperm precedence and its role in reproductive isolation between two subspecies: *Drosophila pseudoobscura pseudoobscura* and *D. p. bogotana*. The final portion of this dissertation examines the rapid evolution of some proteins potentially tied to the evolution of reproductive isolation. I focus on some seminal fluid proteins that may play a role in the reproductive isolation of *Drsosphila* species. In particular, I examine the rapid evolution of accessory gland proteins in the *D. pseudoobscura* subgroup by looking for the signature of positive selection in the genes that encode them. I will also evaluate the roles of insertion / deletion mutations in the evolution of these proteins. Together, the chapters of this dissertation contribute to the

understanding of three forms of prezygotic reproductive isolation and their roles in speciation.

CHAPTER ONE:

INTRODUCTION

Darwin, in *The Origin of Species* (1859), recognized speciation as the driving force behind the diversity of life and the process of speciation has intrigued biologists ever since. Speciation, the splitting of one species into two, occurs when two populations can no longer exchange genetic material (Mayr 1963). The initial steps toward speciation are generally thought to occur in allopatry (when populations are geographically isolated) (Mayr 1963). For example, a geographic barrier such as a mountain range may subdivide an ancestral species, giving rise to two daughter populations to either side of the barrier that will diverge over time due to natural selection and genetic drift (Mayr 1963; Coyne and Orr 1989).

Potentially reproducing populations may evolve numerous barriers that prevent the interbreeding of such incipient species when they come back into contact with each other. Premating isolation barriers operate before mismatings occur. These include mating discrimination, in which species-specific courtship rituals ensure that only conspecific individuals mate (e.g., Noor 1995; Rundle and Schluter 1998). If interspecific mating does occur, postmating / prezygotic isolating barriers may prevent the formation of an unfit hybrid zygote. For example, sperm competition favoring homospecific sperm may ensure that the female's ova are fertilized by the sperm of her same species (e.g. Howard 1993; Price 1997; Chang 2004). Finally, in the event of a successful mismating and the formation of a zygote, postzygotic isolation barriers are present in the form of inviable or infertile offspring (reviewed in Orr and Presgraves 2000; Orr *et al.* 2004).

Such barriers to gene flow may evolve at different points in the process of speciation. They can arise in allopatry (when newly forming species are isolated) or in sympatry (when they co-occur). Lande (1982) showed geographic differentiation in male

traits could be accelerated by the evolution of female preferences. When the two populations are no longer geographically isolated, they would be unable to exchange genes due to the fact that they evolved reproductive isolation during allopatry. Alternatively, under Dobzhansky's model of reinforcement (1940), discrimination can be genetically reinforced to prevent the formation of maladaptive hybrids. Here, barriers that complete reproductive isolation evolve in sympatry in response to the formation of unfit hybrids. This process of reinforcement has been documented in a wide range of taxa (see reviews in Howard 1993; Noor 1999; Servedio and Noor 2004). Reinforcement predicts that females derived from populations of overlap between species will exhibit stronger mating discrimination than those from populations where the two species do not overlap.

This dissertation will examine prezygotic isolation (i.e., barriers to gene exchange that occur before zygotes are formed). Specifically, it will address the reinforcement of mating discrimination (premating isolation), sperm precedence, and the rapid evolution of seminal fluid proteins (the latter two both being forms of postmating / prezygotic isolation).

Premating isolation can be classified into four categories: ecological isolation, temporal isolation, mechanical isolation, and sexual isolation (Dobzhansky 1951). Ecological isolation results when individuals of populations occur in different habitats and thus will not encounter one another. In temporal (or seasonal) isolation, the reproduction times of populations do not coincide (i.e., mating occurs at different times of the year or even different times of the day). Mechanical isolation occurs when reproductive structures are not compatible between species; for example, when genitals are incompatible or when plants have different pollinators.

This dissertation focuses on premating barriers where gene flow between populations is prevented due to sexual isolation. One example of sexual isolation is mating discrimination; females from one species are unreceptive to courtship by males of another species. Theory predicts that sexual selection can result in rapid evolution and may serve as a driving force for speciation (West-Eberhard 1983).

Before mating, males and females exchange many signals that may be visual, chemical, or acoustic (Ewing 1983; Cobb and Ferveur 1996). This is exemplified in the courtship ritual shared by many species of *Drosophila* (reviewed in Hall 1994; see Figure 1.1). First, the male orients toward the head of the female. He taps her abdomen with his foreleg, and the pair exchange species-specific cuticular hydrocarbons (pheromones). Next, the male extends his wing and vibrates it to produce a species-specific courtship song. He then licks her genitalia and mounts her to attempt copulation. A female may reject a male by kicking him with her hind legs and/or fluttering her wings. If she does

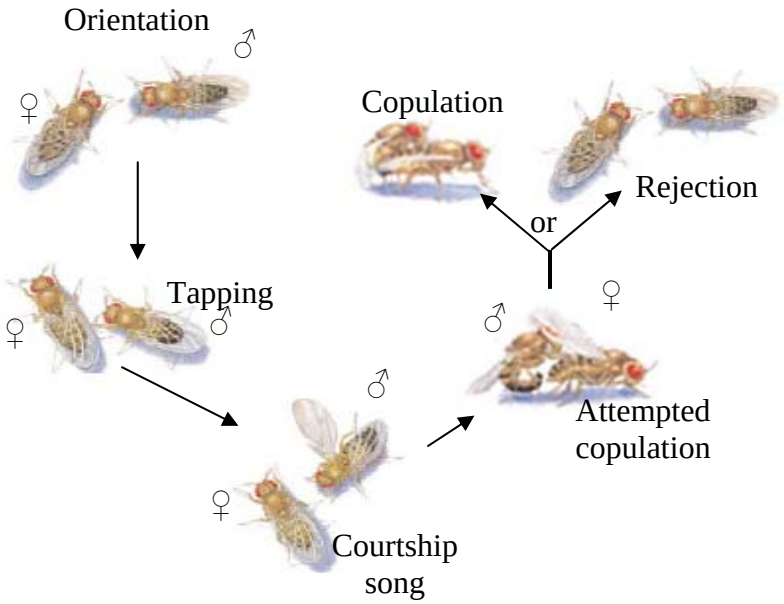

Figure 1.1. Courtship ritual in *Drosophila*. The mating ritual exhibited by *Drosophila* species is detailed in the text.

not reject him, copulation occurs. If the copulation attempt fails, the male will attempt repeat the courtship. Miscommunications in these signals may prevent copulation acceptance by the female.

Although premating reproductive isolation has evolved in many taxa, mismatings between species still commonly occur in the wild. Consequently, many species have evolved postmating / prezygotic barriers to gene exchange. These barriers occur after mating and the transfer of male gametes, but before a zygote has formed. One of the most studied forms of postmating / prezygotic isolation is gametic isolation, which can be either noncompetitive or competitive. Noncompetitive gametic isolation occurs when there are problems with sperm transfer, sperm storage, or fertilization between members of different species (Price *et al.* 2001). This has been demonstrated between the sister species *Drosophila yakuba* and *D. santomea*. Matings between these two species produce significantly fewer offspring than matings within species (Chang 2004). Competitive gametic isolation occurs when one species' gametes are not properly transferred, stored or used when in competition with the other species' gametes. The most prevalent form of competitive gametic isolation is sperm competiton, "the competition between the sperm of two or more males for the fertilization of a given set of ova" (Parker 1970; see also Smith 1984).

Females of most animal species mate multiply, often with different males (Arnquist and Nilson 2000). Female remating is an important component of *Drosophila* mating systems because females store large numbers of sperm after mating in two sac-like organs termed spermathecae and in the seminal receptacle (Miller 1950; Pitnick *et al.* 1999; Tram and Wolfner 1999). Here, they can utilize the sperm for up to two weeks to fertilize

eggs as they are laid (Wolfner 1997). It is in these storage organs that sperm from multiple males mix, thus setting the stage for sperm competition.

Generally, sperm from the last male to mate takes precedence over those of previous males (e.g., Gromko *et al.* 1984; Smith 1984). However, when a female is mated to both conspecific and heterospecific males, she will preferentially produce conspecific rather than hybrid offspring, regardless of the order of matings (Howard 1998). For example, when a *D. simulans* female is mated both with a conspecific and heterospecific male (*D. sechellia* or *D. mauritiana*), the conspecific male's sperm fertilize a majority of the female's eggs regardless of mating order (Price 1997). This phenomenon, termed conspecific sperm precedence, can play a major role in reproductive isolation between two closely related taxa.

The mechanism(s) responsible for conspecific sperm precedence are unknown. Theoretically, conspecific sperm competition may be the result of both noncompetitive and competitive gametic isolation. For example, the sperm of heterospecific males may not be stored properly in the female's storage organs. Conversely, the sperm from conspecific males may out-compete the sperm of heterospecific males. In either case, the conspecific male's sperm will fertilize more eggs than the heterospecific sperm ensuring that more pure species offspring are produced.

Whatever the exact mechanism of prezygotic isolation may be, ultimately all these processes must be mediated by species-specific reproductive proteins. For example, in sea urchins, the sperm protein bindin and the complementary receptors of the egg have coevolved such that the bindin of one species often does not recognize the bindin

receptors on the oocytes of other species. Sperm thus cannot attach to heterospecific eggs, and prezygotic isolation results (Palumbi and Metz 1991; Metz *et al.* 1994).

Another widely studied group of proteins that are associated with reproduction are the Accessory gland proteins (Acps) of *Drosophila*. These proteins are produced by the male accessory gland and are passed into females in the seminal fluid that accompanies the sperm. Different Acps elicit a wide range of behavioral and physiological changes in the mated female (Wolfner 1997), including increasing egg-laying rate (Herndon and Wolfner 1995; Heifetz *et al.*2000; Chapman *et al.*2001; Heifetz *et al.*2001), promoting sperm storage (Neubaum and Wolfner 1999; Tram and Wolfner 1999; Xue and Noll 2000), reducing female willingness to remate (Chen *et al.*1988; Aigaki *et al.*1991), reducing female lifespan (Chapman *et al.*1995; Lung *et al.*2002), and mediating sperm competition (Harshman and Prout 1994; Clark *et al.*1995).

Acps, along with many other proteins involved in reproduction, often undergo accelerated rates of evolution compared to non-reproductive proteins (e.g., Civetta and Singh 1999; Singh and Kulathinal 2000; Vacquier 1998). The rapid evolution of reproductive proteins is often driven by positive selection, which promotes the evolution of amino acid changes (reviewed in Swanson and Vacquier 2002). Positive selection can be identified by comparing the nonsynonymous to synonymous substitution rates (dN/dS) for protein coding regions between closely related taxa. Positively selected genes will have a dN/dS value greater than one (Hughes and Nei 1988; Hughes and Nei 1989). The rapid divergence of reproductive proteins can cause barriers to fertilization that will lead to reproductive isolation and ultimately speciation.

In this dissertation, I aim to evaluate reproductive isolation on several levels. To do

so, I required a study system that displays premating isolation, postmating / prezygotic isolation and postzygotic isolation. The widely studied genetics and geographic distribution of *Drosophila pseudoobscura*, its close relatives (*D. persimilis* and *D. miranda*) and its subspecies (*D. p. bogotana*), offer an excellent system to address reproductive isolation. Although much work has been done regarding postzygotic reproductive isolation (e.g., Orr 1987; Orr and Irving 2001) and reinforcement (e.g. Ortíz-Barrientos *et al.* 2004) in this group, the evolution of prezygotic isolation in this group needs to be dissected.

Drosophila pseudoobscura and *D. persimilis* are thought to have diverged ~500,000 years ago (Figure 1.2) (Aquadro *et al.* 1991). These species are morphologically identical, but can be distinguished based on different chromosomal arrangements. These species exhibit nearly complete sexual isolation (Dobzhansky and Epling 1944); females of both species discriminate against heterospecific males while males court females of either species indiscriminately (Mayr 1946; Noor 1996) and hybridize rarely in the wild. Interspecific matings between these species produce sterile males but fertile females. However, some degree of gene flow continues between these species (Powell 1983; Wang *et al.* 1997).

Drosophila pseudoobscura and its subspecies *D. p. bogotana* diverged approximately 150,000 years ago (Figure 1.2) (Aquadro *et al.* 1991). Reproductive isolation exists between these allopatric subspecies, but is not complete: female *D. p. bogotana* crossed to male *D. pseudoobscura* give rise to sterile males and fertile females (Prakash 1972), while crosses with *D. pseudoobscura* females give all fertile offspring.

Reproductive isolation between the outgroup, *D. miranda*, and its sibling species is essentially complete (Dobzhansky and Epling 1944).

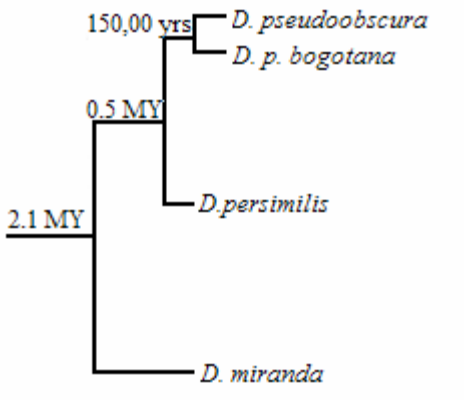

Figure 1.2. Phylogenic relationships and divergence times of members of the *D. pseudoobscura* subgroup. Divergence times are based on the *amylase* gene and are from Aquadro *et al.* 1991.

The ranges of *D. persimilis* and *D. miranda* are contained within the range of *D. pseudoobscura*, and are found in the mountain ranges along the Pacific coast. *D. pseudoobscura*'s range extends from British Colombia southward along the western portion of the United States and into Mexico. *D. p. bogotana* is completely allopatric to *D. pseudoobscura*, *D. persimilis* and *D. miranda* and is isolated to areas surrounding Bogotá, Colombia. This study system thus allows for the evaluation of prezygotic reproductive isolation acting at different stages of divergence both within and outside of sympatry.

The objective of this dissertation is to evaluate prezygotic reproductive isolation in the *D. pseudoobscura* subgroup. I will begin by discussing premating reproductive

isolation through evaluating the mode of evolution that leads to reinforcement in *Drosophila pseudoobscura*. In particular, I will evaluate two alternative hypotheses; Preference Evolution and Discrimination Enhancement, to determine how selection reduces hybridization between *D. pseudoobscura* and *D. persimilis*. Both hypotheses predict a reduction in the overlap of male traits and female preferences in hybridizing populations, thus causing reinforcement; however, the target of selection differs between the two. I will examine these alternative hypotheses in the context of reinforcement in *Drosophila pseudoobscura* and *D. persimilis*. Next, in chapter three, I will discuss reproductive isolation as a result of competiton between gametes of individuals, in particular conspecific sperm precedence. Until this study, patterns of sperm precedence had rarely been examined between divergent populations or subspecies within a species. I will evaluate conspecific sperm precedence and its role in reproductive isolation between two subspecies: *Drosophila pseudoobscura pseudoobscura* and *D. p. bogotana*. The final portion of this dissertation is devoted to evaluating reproductive isolation as a result of rapid protein divergence. Chapter four focuses on some seminal fluid proteins that may play a role in the reproductive isolation of species. In particular, I will examine the rapid evolution of accessory gland proteins in the *D. pseudoobscura* subgroup by looking for the signature of positive selection in the genes that encode them. I will also evaluate the role of insertion / deletion mutations in the evolution of these proteins. Together, the chapters of this dissertation provide relevant data toward understanding three forms of prezygotic reproductive isolation and their roles in speciation.

CHAPTER TWO*:

EVALUATING THE MODE OF REINFORCEMENT IN *DROSOPHILA PSEUDOOBSCURA*: DISCRIMINATION ENHANCEMENT VERSUS PREFERENCE EVOLUTION

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INTRODUCTION

Reinforcement is the process by which natural selection increases premating reproductive isolation (e.g., mating discrimination) to prevent maladaptive hybridization. For example, if two species have overlapping geographic ranges, and if these species do not mate exclusively with conspecifics, sterile hybrids may be produced. Producing sterile hybrids imposes a cost on these species, and any variation allowing preferential mating with conspecifics will be favored by natural selection in the regions of geographic overlap.

This leads to a pattern of "reproductive character displacement": individuals derived from populations of overlap between species (sympatry) will exhibit strong mating discrimination while those from other populations (allopatry) may exhibit weaker mating discrimination. This process has been documented in a wide range of taxa (see reviews in Howard 1993; Noor 1999; Servedio and Noor 2004). Less clear, however, is how selection reduces hybridization. Some theoretical models (e.g., Lande 1981; Liou and Price 1994) posited that reinforcement occurs by divergence of the distribution of female preferences (see Figure 2.1). Females exhibiting preferences for extreme traits that are only present in males of one species are favored, so the entire female preference distribution shifts in populations of geographic overlap.

Concomitant with this, males exhibiting extreme traits are also favored, and the distribution of male traits is expected to coevolve in parallel (e.g., Ritchie 1996). We call this scenario "preference evolution." An alternative scenario is that females increase discrimination through reducing the range of characters with which they are willing to mate (Kelly and Noor 1996). The outcome of this process would be a reduction in

overlap of female preferences between the two species, followed by a concomitant reduction in overlap of male traits. We call this scenario "discrimination enhancement." Both scenarios reduce the overlap of male traits and female preferences in hybridizing populations, hence causing reinforcement. However, the target of selection differs in the two: in preference evolution, the primary change is a directional shift in the distribution of female preferences; while in discrimination enhancement, the primary change is a reduction in the variance or breadth of such a distribution. Distinguishing these hypotheses can have a great impact on determining the likelihood of speciation by reinforcement.

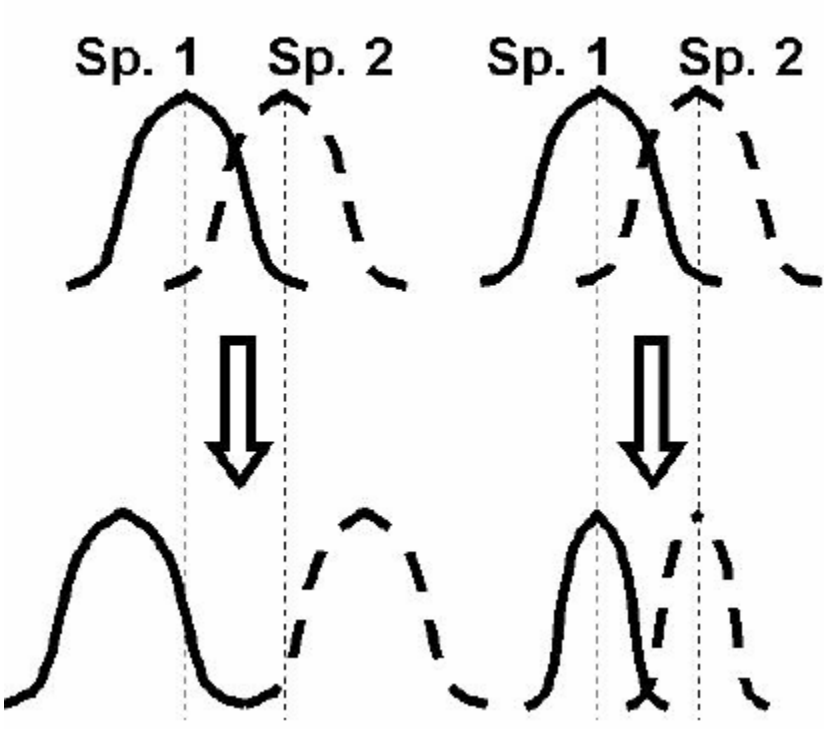

Figure 2.1. Alternative models for the mode of reinforcement in *Drosophila*. Sympatric species female preference function change under preference evolution (left), with a shift in the distribution of preferences, vs. discrimination enhancement (right), with a narrowing in the breadth of female preferences.

Several predictions distinguish these models. First, the preference evolution hypothesis predicts partial sexual isolation among populations within species, as divergence in the distribution of female preferences should only occur in populations that co-occur with heterospecifics. Discrimination enhancement makes no such prediction, as most males and females in the separated populations within species should be phenotypically and behaviorally similar. Second, preference evolution predicts that heterospecific females should prefer males from nonoverlapping populations relative to those from overlapping populations. In contrast, discrimination enhancement predicts no difference in how heterospecific females perceive males from different populations, as again, they should be phenotypically similar.

We examine these hypotheses in the context of reinforcement in *Drosophila pseudoobscura* and *D. persimilis*. These species overlap in western North America, hybridize rarely, and exhibit reproductive character displacement in female preference (Noor 1995). Consistent with the discrimination enhancement model, Anderson and Ehrman (1969) observed no mating discrimination among populations of *D. pseudoobscura*. Here, I test the second prediction of discrimination enhancement: whether *D. persimilis* females prefer *D. pseudoobscura* males from nonoverlapping (allopatric) populations. Consistent with the discrimination enhancement model, we find that they do not.

MATERIALS AND METHODS

Flies used in the mating experiments were reared at 20 ± 1 °C, 85% relative humidity, 12:12 hour light: dark cycle, on standard sugar/agar/yeast medium. Bottles were cleared of adults before incubator lights came on and virgin adults collected less

than seven hours later under CO2 anesthesia. Virgin flies were separated by sex and stored in vials containing food for seven days. One day prior to mating, males were separated and stored in vials individually to reduce crowding-mediated courtship inhibition (Noor 1997). On the eighth day, single females were aspirated into vials with single males for mating observations.

We performed the experiment using inbred *D. pseudoobscura* lines and using outbred F1 progeny from crosses between inbred *D. pseudoobscura* lines. The *D. persimilis* line used was collected in Mount St. Helena (MSH), California, in 1993. The inbred *D. pseudoobscura* lines used were Mather, California number 17 (collected 1997) and Flagstaff, Arizona (collected 1993). The *D. pseudoobscura* lines crossed for the outbred experiments were Flagstaff lines 6 and 14 (collected 2001) and Mount St. Helena lines 12 and 17 (collected 2001). The California lines are from areas of species coexistence (sympatry), while *D. pseudoobscura* is found alone in Arizona (allopatry).

We first confirmed the pattern of reproductive character displacement in female *D. pseudoobscura*, we examined mate preferences of Arizona vs. California *D. pseudoobscura* females when paired with *D. persimilis* males. We anticipate that the females derived from California would exhibit the greater reluctance to mate with *D. persimilis* males (Noor 1995). Flies were paired singly in food vials and observed for 10 minutes after onset of male courtship for mating (no-choice mating design). For our test of the discrimination enhancement model, we paired *D. persimilis* females with *D. pseudoobscura* males singly (no-choice mating design) and observed them for 10 minutes after onset of male courtship. Statistical analyses used Fisher's exact tests as executed on StatView®.

RESULTS

Consistent with reproductive character displacement in female preferences, *D. pseudoobscura* females derived from sympatric populations in California were more reluctant than *D. pseudoobscura* females from Arizona to mate with *D. persimilis* males (Table 2.1). This was true both for the inbred and the outbred lines tested. In contrast, *D. persimilis* females exhibited no preference for *D. pseudoobscura* males from Arizona vs. California populations.

Table 2.1. No-choice mating experiment results involving crosses between *D. persimilis* (per) and *D. pseudoobscura* (ps).

Female	Male	% Mated	N	p
ps Mather 17	per MSH 1993	11.3	106	<0.0001
ps Flagstaff 1993	per MSH 1993	41.5	106	
ps MSH 12 x 7	per MSH 1993	12.0	100	0.0279
ps Flagstaff 6 x 14	per MSH 1993	25.0	100	
per MSH 1993	ps Mather 17	51.9	106	0.680
per MSH 1993	ps Flagstaff 1993	55.7	106	
per MSH 1993	ps MSH 12 x 7	37.0	100	0.314
per MSH 1993	ps Flagstaff 6 x 14	45.0	100	

DISCUSSION

Using mate preference experiments with *D. pseudoobscura* and *D. persimilis*, we present an explicit test of two models of speciation by reinforcement: preference evolution vs. discrimination enhancement. Both models predict that females derived from

populations co-occurring with heterospecifics will exhibit greater mate discrimination than females derived from populations where no heterospecifics exist (reproductive character displacement). The preference evolution model further predicts that females should prefer to mate with heterospecific males from populations where conspecific males do not occur over heterospecific males from populations where conspecific males do occur. The discrimination enhancement makes no such prediction.

Our results are consistent with the discrimination enhancement model of reinforcement: while we did detect the signature of reproductive character displacement, we failed to observe a preference by females for heterospecific males from allopatric populations. Other data on this species pair also fails to provide evidence for the other prediction of preference evolution: that some weak mating discrimination should be observed against individuals from other populations (Anderson and Ehrman 1969).

Discrimination enhancement may be a common mode by which reinforcement occurs. For example, Butlin (1993) showed that, in the brown planthopper *Nilaparvata lugens*, there was greater variation in the width of female preference functions than in mean female preference. Hence, if natural selection were to reduce overlap in female preferences between two species, it would likely do so through increasing discrimination rather than shifting the mean female preference.

Reinforcement was once a controversial mode of speciation, but empirical studies have provided evidence for its existence and theoretical studies have suggested specific conditions under which it may be particularly likely (see reviews in Noor 1999; Servedio and Noor 2003; Coyne and Orr 2004). As the discrimination enhancement model was suggested as a likely means in which it could occur (Kelly and Noor 1996), and as we

provide empirical data consistent with this model in the *D. pseudoobscura* group, it merits further empirical investigation and confirmation in other taxa.

CHAPTER THREE*:

THE EVOLUTION OF CONSPCIFIC SPERM PRECEDENCE IN *DROSOPHILA*

*This chapter along with additional data was published in *Molecular Ecology*. It has been reprinted with permission from *Molecular Ecology*.

INTRODUCTION

Barriers to gene exchange between closely related taxa, including those that operate via either premating or postzygotic mechanisms, are thought to be those that cause speciation. Researchers have recently begun to study barriers to gene exchange that operate after mating but before zygotes are formed (Howard 1999). One of the most notable postmating/ prezygotic barriers is conspecific sperm precedence. Conspecific sperm precedence takes place when females inseminated by both conspecific and heterospecific sperm preferentially produce conspecific rather than hybrid offspring. Postinsemination sperm success has been studied generally in grasshoppers (Hewitt *et al.* 1989; Bella *et al.* 1992), *Drosophila* (e.g., Civetta and Clark 2000; Price *et al.* 2000; Snook and Markow 2002), crickets (Howard and Gregory 1993; Gregory and Howard 1994; Howard *et al.* 1998), flour beetles (Wade *et al.* 1994; Lewis and Jutkiewicz 1998), and several plant species (as conspecific pollen precedence, see e.g., Rieseberg *et al.* 1995; Carney *et al.* 1996). Several of these studies have identified patterns of fertilization consistent with conspecific sperm precedence, but researchers have yet to determine how rapidly conspecific sperm precedence evolves. Several authors (e.g., Howard 1999) suggest that it evolves early in evolutionary divergence and may frequently contribute to speciation. However, most studies have identified sperm precedence only between taxa that are considered to be good species based on possessing other barriers to gene exchange (but see Bella *et al.* 1992; Gregory and Howard 1994). Patterns of sperm precedence have rarely been examined between divergent populations or subspecies within a species.

In matings within most *Drosophila* species, as well as some other taxa, the last male to copulate sires most of the offspring (e.g., Gromko *et al.* 1984; Smith 1984). In contrast, when a *D. simulans* female is mated both with a conspecific and heterospecific male (*D. sechellia* or *D. mauritiana*), the conspecific male's sperm fertilize a majority of the female's eggs regardless of mating order (Price 1997): evidence for conspecific sperm precedence. The high proportion of offspring sired by the conspecific male when mated first indicates conspecific sperm precedence exists in these species. Here, we test for evidence of the early stages of conspecific sperm precedence ("contypic sperm precedence") between two *Drosophila* subspecies (*Drosophila pseudoobscura pseudoobscura* and *D. p. bogotana*). If conspecific sperm precedence always contributes to speciation, it should evolve before the reproductive isolation of two taxa is complete.

MATERIALS AND METHODS

Strains

The strains of flies used in this study were *D. pseudoobscura* Flagstaff 1993 (collected in Flagstaff, Arizona, USA, in 1993), *D. pseudoobscura* AFC 12 (collected in American Fork, Utah, USA, in 1997), *D. p. bogotana* Sutatausa 5 (collected in Sutatausa, Colombia, in 1997), *D. p. bogotana* Susa 6 (collected in Susa, Colombia, in 1997).

Handling and Mating

Flies to be used in the mating experiments were reared at 21±1°C, 85% relative humidity, on standard sugar/ yeast/ agar medium. Bottles were cleared and virgin adults collected less than seven hours later under CO_2 anesthesia. Flies were separated by sex and stored in vials containing food for eight days. One day prior to mating, males were

separated and stored in vials individually to reduce crowding mediated courtship inhibition (Noor 1997).

On the eighth day, single females were aspirated into vials with single males. Some females were paired first to a contypic male then one week later to a heterotypic male, and other females were paired in the reverse order. Females were allowed to mate only once with a particular male and flies that laid viable eggs from first matings were used for second matings. All attempts to remate females in less than one week were unsuccessful. The males were allowed to court for at least fifteen minutes or until mating and all copulations were observed and timed. All copulations shorter than 60 seconds were excluded. All females produced progeny during the one-week period between first and second matings. Males were removed from the vials and stored at -20°C shortly after mating. Females that had mated with both a heterotypic male and a contypic male were housed individually and offspring were collected as they eclosed. Approximately fifty offspring were collected from each female.

Molecular Markers

DNA was extracted using the protocol of Gloor and Engels (1992). Strains of the two subspecies were differentiated from one another by using a hypervariable microsatellite marker (DPS2005: see Noor *et al.* 2000). The microsatellite was amplified by PCR, and the products visualized and scored on 2% TBE ethidium-bromide stained agarose gels. Paternity was designated by homozygosity versus heterozygosity at DPS2005 in the offspring. The proportion of offspring sired by the second male (P2) was compared between pairings using Mann-Whitney U-tests. We repeated the statistical analyses with square-root transformed data, and all results were identical.

Controls

We performed two sets of controls to test for effects of larval competition or differences in viability on the outcome of our studies. First, a *D. p. pseudoobscura* female mated to a *D. p. pseudoobscura* male and one mated to a *D. p. bogotana* male were placed into the same vial and allowed to lay eggs. Again, females were allowed to mate only once with a male. The offspring from both females were collected as they appeared. The microsatellites of the offspring were amplified again using PCR and scored on 2% agarose gels. Paternity was designated by homozygosity versus heterozygosity at DPS2005 in the offspring. Next, a *D. p. bogotana* female that had mated with a *D. p. pseudoobscura* male was placed into a vial with a *D. p. pseudoobscura* female that had mated with a *D. p. pseudoobscura* male. The offspring from both females were collected as they appeared, and their maternity was scored as described above.

RESULTS

For simplicity, Table 3.1 presents abbreviations that we will use to refer to the mating order of the crosses used in these experiments. Proportions of offspring from each cross sired by the second males (P2) and all other data related to the mating experiments are shown in Table 3.2. In all experiments, we observed that the proportion of offspring sired by the second male (P2) was at least 65% (Table 2). As such, any contypic sperm precedence observed appears to be weak relative to the strong second-male sperm precedence observed in within-species crosses in *Drosophila*. We have thus analyzed our data by comparing P2 values for particular male mating orders across females of the two taxa.

Table 3.1. Abbreviations used to reference particular mating orders throughout the Results section.

Female	1st male	2nd male	Abbreviation
pseudoobscura	*pseudoobscura*	*bogotana*	ppb
bogotana	*pseudoobscura*	*bogotana*	bpb
pseudoobscura	*bogotana*	*pseudoobscura*	pbp
bogotana	*bogotana*	*pseudoobscura*	bbp

Table 3.2. Proportion of offspring sired by the second male (P2) and sample sizes for each cross.

Cross ♀♂1♂2	*bog* strain	*ps* strain	N	1st male copulation duration	2nd male copulation duration	Mean P2
b b p	Sutatausa 5	Flagstaff 1993	29	446 sec	329 sec	0.887
p b p	Sutatausa 5	Flagstaff 1993	21	479 sec	369 sec	1.00
b p b	Sutatausa 5	Flagstaff 1993	11	381 sec	429 sec	0.935
p p b	Sutatausa 5	Flagstaff 1993	14	448sec	375 sec	0.72
b b p	Susa 6	AFC 12	4	323 sec	308 sec	0.874
p b p	Susa 6	AFC 12	5	322 sec	282 sec	0.960
b p b	Susa 6	AFC 12	6	390 sec	226 sec	0.971
p p b	Susa 6	AFC 12	4	436 sec	328 sec	0.724

In the cross pbp using lines from Flagstaff and Sutatausa, all offspring were sired by the *D. p. pseudoobscura* (*ps*) male, but in the cross bbp only 88.7% of the offspring were sired by the *ps* males (P2=0.887). The difference in P2 observed between these crosses is significant (Mann-Whitney U=178.5, p= 0.0133) even though the male mating order was the same. This suggests that the males' sperm success was different inside the reproductive tracts of the different females.

In the cross ppb, 72% of the offspring were sired by the *D. p. bogotana* (*bog*) male (P2=0.72). In the cross bpb, 93.5% of the offspring were sired by the *bog* male (P2=

0.935). We also noted that 10 of the 11 females that produced offspring in this cross produced 100% *bog* offspring. There was one outlier that produced only 28.3% *bog* offspring. Again, these results indicate statistically significant sperm precedence (Mann-Whitney U= 31.00 p= 0.0118) even though the male mating order was the same between these two crosses, again suggesting that the fertilization success was different in the different females' reproductive tracts.

There was no significant association between P2 and copulation time for the first mating, copulation time for the second mating, or the difference between these times within any of the crosses. There was also no difference in copulation duration between *ps* females and males of the two subspecies in either mating order. However, there was a slight difference in copulation times for *bog* females: on average, *bog* males had longer copulation duration than *ps* males during their second matings to *bog* females (Mann-Whitney U=68.00, p= 0.0073).

The experiment was repeated with a smaller sample size of the *D. p. bogotana* Susa 6 and *D. p. pseudoobscura* AFC 12 lines. In the cross ppb, 72.4% of the offspring were sired by the *D. p. bogotana* (*bog*) male (P2=0.724). In the cross bpb, 97.1% of the offspring were sired by the *bog* male (P2= 0.971). This difference is consistent with a pattern of preferential fertilization by the contypic male (Mann-Whitney U= 2.00 p= 0.033) despite the same male mating order. Perhaps due to the small sample size, comparisons of P2 between bbp and pbp crosses exhibited no significant differences (p= 0.46). However, the pattern of preferential fertilization of the eggs by the contypic male is in the same direction as the Sutatausa/ Flagstaff bbp and pbp crosses listed above.

Controls

Of the offspring collected from control vials in which a *ps* female had mated with a *ps* male and another *ps* female had mated with a *bog* male, 0.556±0.026 (mean ± SE) of the total offspring were hybrids. Of the offspring collected from control vials where a *ps* female and a *bog* female had mated with *ps* males, 0.552±0.107 (mean ± SE) of the total were hybrids. If there was no difference in viability or larval competitive ability between *ps* and hybrid larvae, we expect half of the offspring to be hybrids and this is very close to what we observed. If anything, slightly more hybrids were observed than expected, so hybrid larvae were at least as viable as pure species larvae.

Wolbachia Test

Wolbachia are intercellular parasites that are transferred from infected females to their progeny and can cause cytoplasmic incompatibilities between populations (Laven 1951; Laven 1967) or recently diverged species (Breeuwer and Warren 1990; Breeuwer *et al.* 1992). Since the presence of *Wolbachia* could produce a pattern similar to that seen by contypic sperm precedence, we tested for *Wolbachia* in these taxa. We used primers to amplify *Wolbachia* genes *wsp* and *ftsZ* via long PCR (described by Jeyaprakash and Hoy 2000) from all strains used in this study as well as a strain of *Drosophila simulans* known to be infected with *Wolbachia.* The *D. pseudoobscura* lines did not appear to be infected with *Wolbachia*. These results suggest that cytoplasmic incompatibility in any of our pairings was unlikely, as also indicated by our controls.

DISCUSSION

In the double matings involving *Drosophila pseudoobscura* subspecies, we observed that second-mating males typically sired most of the offspring, but this effect was most pronounced when the second male was of the same subspecies as the female.

The significant preferential fertilization by contypic males suggests that conspecific sperm precedence (CSP) may be in the early stages of the evolution in these subspecies.

Howard (1999), Price (1997), and others have suggested that conspecific sperm precedence evolves early in evolutionary divergence and may evolve before other barriers to gene exchange. Clearly, CSP can evolve before other barriers to gene exchange, as illustrated by the case of *Allonemobius fasciatus* and *A. socius* crickets (Gregory and Howard 1994; Howard and Gregory 1993), which are isolated only by CSP. However, to date, most studies have identified sperm precedence between species possessing several other barriers to gene exchange. Here, by using recently diverged taxa (i.e. subspecies and populations), we demonstrated the overall rate of evolution of conspecific sperm precedence may sometimes be comparable to other barriers to gene exchange, in contrast to observations in *Allonemobius* species.

Between the two allopatric subspecies of *D. pseudoobscura*, conspecific sperm precedence seems to be a weak but significant factor contributing to postmating fertilization success. However, these subspecies already possess complete one-way hybrid male sterility and weak mating discrimination (Noor and Coyne 1995). As such, CSP is evolving at a rate similar to or possibly even slower than the other barriers to gene exchange.

We conclude that conspecific sperm precedence can be an important barrier to gene exchange between taxa, but it does not always evolve before other such barriers such as hybrid sterility or behavioral mating discrimination. It may be an outcome of either sexual conflict, whereby adaptations in one sex decrease the fitness of the other (Birkhead 2000; Chippindale *et al.* 2001) leading to antagonistic coevolution between the

sexes (Arnqvist and Rowe 1995; Rice 1996; Rice 1998), and it may affect the subsequent reinforcement of behavioral barriers to gene exchange that prevent formation of maladapted hybrids (e.g., Marshall *et al.* 2002). Further research to evaluate such hypotheses should focus on the relative rates of evolution of CSP in hybridizing and nonhybridizing species and those bearing strong premating barriers to gene exchange versus those without.

CHAPTER FOUR:

POSITIVE SELECTION ON NUCLEOTIDE SUBSTITUTIONS AND INDELS IN ACCESSORY GLAND PROTEINS OF THE *DROSOPHILA PSEUDOOBSCURA* SUBGROUP

INTRODUCTION

Studies comparing reproductive proteins within and between closely related taxa have often found that genes encoding reproductive proteins are more divergent than genes encoding non-reproductive proteins (e.g., Civetta and Singh 1999; Singh and Kulathinal 2000; Vacquier 1998). This divergence is often due to selection for nucleotide substitutions that result in amino acid changes (Swanson and Vacquier 2002). Such positive selection can be identified by comparing relative rates of nonsynonymous and synonymous changes at orthologous loci. For example, under neutrality the proportion of nonsynonymous to synonymous changes within and between species should be equivalent; departures suggest non-neutral evolution (McDonald and Kreitman 1991). Alternatively, the signature of positive selection can be identified by comparing the ratio of nonsynonymous to synonymous substitution rates (dN/dS, or ω). Positively selected genes will have dN/dS values greater than one (Hughes and Nei 1988; Hughes and Nei 1989).

These methods of detecting selection are conservative when applied over the full coding region of a gene, in that they do not consider variation in selective constraints among codon positions. This can mask the signature of positive selection because homologous proteins that maintain similar functions will generally include conserved domains maintained by stabilizing selection, whose ω should be far less than one. Yang and Neilsen (2000) developed codon-specific models that allow for the identification of specific residues targeted by positive selection using Likelihood Ratio Tests (LTRs). In recent years, such site-specific models have been used to detect positive selection in a variety of genes and species. The power of these sequence-based tests has been verified

by their ability to identify residues already functionally implicated as under positive selection (e.g., Yang and Swanson 2002; Mondragon-Palomino *et al.* 2002).

Protein divergence, however, is not brought about solely by nucleotide substitutions. For example, in the abalone vitelline envelope for lysin (VERL), rapid changes are driven by concerted evolution (Swanson and Vaquier 1998). While Galindo *et al.* (2003) found positive selection at the 5' end of VERL the majority of sites within VERL are not undergoing positive selection, and yet still rapidly evolve (Swanson *et al.* 2001a). Insertions and deletions (indels) are another potential source of variation upon which positive selection may act. Indels occur as frequently as nucleotide substitutions throughout the genome (Britten *et al.* 2003; Denver *et al.* 2004), and recent studies have shown positive selection acting on indels in sperm-specific proteins in mammals (Podlaha and Zhang 2003; Podlaha *et al.* 2005).

Here, we evaluate some of the best characterized examples of rapid divergence in reproductive proteins: the accessory gland proteins (Acps) of *Drosophila*. During mating, *D. melanogaster* males transfer approximately 83 Acps to females in the seminal fluid that accompanies sperm (Chen *et al.* 1988; Swanson *et al.* 2001a). These Acps elicit many behavioral and physiological changes in the mated female (Wolfner 2002), including increasing egg-laying rate (Herndon and Wolfner 1995; Heifetz *et al.* 2000; Chapman *et al.* 2001; Heifetz *et al.* 2001), promoting sperm storage (Neubaum and Wolfner 1999; Tram and Wolfner 1999; Xue and Noll 2000), reducing female willingness to remate (Chen *et al.* 1988; Aigaki *et al.* 1991), reducing female lifespan (Chapman *et al.* 1995; Lung *et al.* 2002), and mediating sperm competition (Harshman and Prout 1994; Clark *et al.* 1995). Studies have shown that Acps in this subgroup are on

average twice as divergent between species as non-reproductive proteins (Civetta and Singh 1995; Singh and Kulathinal 2000).

While the functions of and selection on Acps in the *D. melanogaster* subgroup have been widely studied, little is known about Acps in other drosophilid lineages. The recent publication of the *D. pseudoobscura* genome (Richards *et al.* 2005) permits the comparison of Acp evolution within lineages that have been independent for 21-46 MYA (Beckenbach *et al.* 1993). Wagstaff and Begun (2005) used a combination of computational and molecular approaches to identify five orthologous Acp loci from the *D. melanogaster* group in *D. pseudoobscura*: *Acp26Aa, Acp32CD, Acp53Ea, Acp62F,* and *Acp70A.* The function(s) of these Acps in *D. melanogaster* are listed in Table 4.1.

Table 4.1. Accessory gland protein functions. Accessory gland proteins used in this study and their function(s) in *D. melanogaster*

Protein	Function(s) in *D. melanogaster*
Acp26Aa	Hormonal activity; Increases egg-laying (Herndon and Wolfner 1995; Heifetz *et al.* 2000; Chapman *et al.* 2001; Heifetz *et al.* 2001); Involved in sperm competition (Clark *et al.* 1995)
Acp32CD	Function unknown (M. Wolfner, personal communication, June 2005)
Acp53Ea	Hormonal activity; Involved in sperm competition (Clark *et al.* 1995)
Acp62F	Protects sperm from proteolysis (Lung *et al.* 2002); Decreases female's life span (Chapman *et al.* 1995; Lung *et al.* 2002)
Acp70A (sex peptide)	Hormonal activity; Increases egg-laying (Chen *et al.* 1988; Aigaki *et al.* 1991; Soller *et al.* 1997, 1999); Decreases female receptivity (Chen *et al.* 1988; Aigaki *et al.* 1991)

Here, we inspect for the signature of positive selection of these five Acps in the *D. pseudoobscura* subgroup, and then compare relative rates of change in this clade to those in the *D. melanogaster* subgroup. If the patterns of molecular evolution in these Acps are similar between these two clades, it would suggest that the conserved functions of these

proteins remain a constant target of selection over large time scales. Alternatively, different patterns of selection on orthologous reproductive proteins in the two lineages would suggest that different loci might provide opportunistic targets for selection at different points in the phylogeny. In addition to nucleotide substitution rates, we evaluate the role that indels, a source of variation heretofore ignored in studies of Acps in *Drosophila*, play in the divergence of these proteins.

MATERIALS AND METHODS

Fly Stocks

Flies used in this study were obtained from Dr. Mohamed Noor, Dr. Carlos Machado, and the Tucson Stock center (http://stockcenter.arl.arizona.edu), and largely overlap with those used in Machado *et al.* (2002). We used 20 lines of *D. pseudoobscura*: four lines from Mather, California (Mather17, Mather32, Mather52, and Mather1959); four lines from Mt. St. Helena, California (MSH9, MSH21, MSH24, and MSH32); one line from James Reserve, California; four lines from American Fort Canyon, Utah (AF2, AFC3, AFC7, and AFC12); four lines from Flagstaff, Arizona (Flagstaff5, Flagstaff14, Flagstaff16 and Flagstaff18); one line from Tucson, Arizona; one line from Baja, California (Baja 1); and one line from Sonora, Mexico (Sonora 3). We also used eleven lines of *D. p. bogotana* from near the city of Bogotá in Cundinamarca, Colombia (Bogotá 1960, Bogotá 1976, Potosý´2, Potosý´3, Susa2, Susa6, Sutatausa3, Sutatausa5, Toro1, Toro6, and Toro7), seven lines of *D. persimilis*: three lines from Mather, California (Mather37, Mather40, MatherG) and four lines from Mt. St. Helena, CA (MSH1, MSH3, MSH7, and MSH42), and three lines of *D. miranda* (MSH22, MSH38, and Mather 1993).

DNA Isolation, PCR Amplification, and Sequencing

DNA was extracted from whole male flies using the single fly squish protocol of Gloor and Engels (1992). PCR primers were designed from the *D. pseudoobscura* Acp sequences *Acp26Aa*, *Acp32CD*, *Acp53Ea*, *Acp62F*, and *Acp70A* from Wagstaff and Begun (2005) using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3), and are listed in Table 4.2. The PCR was performed on a PTC-200 (MJ Research, Watertown, MA) with the following conditions: 94°C for 2 minutes and 30 seconds, 50°C for 2 minutes then 72°C for 2 minutes followed by 38 cycles of 94°C for 45 seconds, 50°C for 1 minute then 72°C for 1 minute and 15 seconds. Resulting amplicons were purified using either a Strataprep ® PCR Purification Kit (Stratagene, La Jolla, CA) or QuickStep™2 96-Well PCR Purification Kit (Edge BioSystems, Gaithersburg, MD), then sequenced in both directions on an ABI 377 automated sequencer, using Big Dye Terminators (V3.1, Applied Biosystems, Foster City, CA) and the amplification primers. Sequences will be submitted to the GenBank database.

Table 4.2. List of primer sequences used to amplify Acps in the *D. pseudoobscura* group.

Gene	Primer sequences
Acp26Aa	F: CAGAAGATGATCCCCCAAAG R: CCATTTCAAGTTCGTGACAGC
Acp32CD	F: CCAAAGCTTGGGATTGTAGC R: TTCAACCTCCGAAACTCCAC
Acp53Ea	F: GCAGTGCATGCTATCAATCC R: AAGACAGAGAAAGCCCGAAA
Acp62F	F: CTATCGCATAAATTCCCACAGAAC R: ACCAACAAACACTTCCAACAGAC
Acp70A	F: CCTCGAACCAGACTCAAAACTC R: TTAAGTACGACTAAGCTGCATCC

Sequence Analyses

Nucleotide sequences for each Acp were initially assembled and edited with Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI). Amino acid sequences were then aligned with ClustalW (http://www2.ebi.ac.uk/clustalw/) under default settings. Further alignment modifications were made by hand. Amino acid alignments were then used to assemble nucleotide alignments. Kimura 2-Parameter distances for the resulting nucleotide datasets were then analyzed using neighbor-joining (NJ) analyses (Saitou and Nei 1987) in PAUP* v4.0b10 (Swofford, 2000). Branch support was estimated by bootstrapping using 1000 replicates. Measures of Acp polymorphism and divergence, as well as McDonald-Kreitman's (1991) test for non-neutrality, were calculated using DnaSP 4.0 (Rozas *et al.* 2003). These measures can reveal positive selection acting across all sites of a protein by comparing the number of silent versus replacement polymorphisms.

Orthologous Acp sequences from the *D. melanogaster* subgroup were downloaded from GenBank. These appeared initially in Tsaur *et al.* 2001 (AF302208–AF302229), Begun *et al.* 2001 (AY010527–AY010711), Panhuis *et al.* 2003 (AY344246–AY344364), Holloway and Begun 2004 (AY635196-AY635290) and Kern *et al.* 2004 (AY505178–AY505293). Sequences for analysis were chosen by sequence length (if >75% of the protein's open reading frame was available for download) and uniqueness (identical sequences were not included). For our analyses, *D. melanogaster* (Zimbabwe) and *D. p. bogotana* were considered taxa.

The codeml program in PAML 3.14 (Yang 2004) was used to test for positive selection and to infer amino acid sites under positive selection under the maximum

likelihood methods of Nielson and Yang (1998) and Yang *et al.* (2000). A Bayes Empirical Bayes (BEB;Deely and Lindley 1981) approach, as described in Yang *et al.* (2005), was subsequently used to calculate the posterior probabilities that each particular site fell into the different ω classes (Neilsen and Yang 1998; Yang *et al.* 2000; Yang *et al.* 2005).

We performed two tests for positive selection. First, we used a simple model (M0) that assumed one dN/dS (or ω) for all sites to estimate levels of positive selection averaged over all codons. Second, a more robust test for adaptive evolution was performed by comparing the nested models M7 and M8. The neutral model (M7) allowed ω to take on beta-distributed values between 0 and 1 at each codon (i.e. no positive selection). This was compared with a selection model (M8), which used the same beta-distributed values for neutral codons, but added a parameter that allows a proportion of codons to take on ω values greater than one. Positive selection was inferred by $\omega > 1.0$ and significance was determined by comparing twice the difference between the likelihood values of M8 vs. M7 to a chi-square table of critical values.

We used the BEB method to identify positively selected residues instead of alternative parsimony-based approaches (Suzuki and Nei 2004; Zhang 2004) because: 1) while the parsimony methods have a low rate of false positives, they also have little power for detecting positive selection or identifying positively selected sites (Wang *et al.* 2004), and 2) while older Naive Empirical Bayesian approach (NEB) can have high false-positive rates, the BEB approach corrects for past problems and reduces the false positive rate considerably (Yang *et al.* 2005). Through the BEB approach, sites under positive selection can be identified, even if the average dN/dS over all sites is less than one. Sites

with a high probability of belonging to the class with $\omega > 1$ are likely to be under positive selection.

RESULTS

Intraspecific Variation

The neighbor-joining trees of Acps in the *D. pseudoobscura* subgroup (Figures 4.1-4.5) showed very little divergence between taxa. Many branches had bootstrap support <70%, reflecting a lack of phylogenetically informative changes. For all of the Acps evaluated, *D. miranda* was the outgroup to the other taxa. In most other cases, individuals from the same taxon grouped together, and with the same topology as generally accepted for this group. *Acp26Aa* (Fig. 4.1) provided the strongest exception, with many *D. persimilis* alleles grouping with *D. pseudoobscura* alleles, to the exclusion of a basal group of *D. psuedoobscura* alleles.

Low levels of variation were also evident in both Watterson's and Nei's estimates of nucleotide site diversity (Table 4.3). Watterson's theta (θ) was used to calculate the mutation rate of a population, and serves as a measure of nucleotide variation (Watterson 1975). For both θ_w and Nei's pi (π), *D. pseudoobscura* had the highest levels of nucleotide variation at *Acp26Aa*, *Acp32CD* and *Acp62F*, while *D. miranda* had the highest nucleotide variation for *Acp53Ea* and *Acp70A*.

Tajima's D (Tajima 1989) was used as one test for whether the patterns of nucleotide variation were consistent with the neutral model. This statistic was not significantly different from zero in any taxon within the *D. pseudoobscura* group for any of the Acp loci (Table 4.3). Therefore, we cannot reject the hypothesis that these loci are evolving neutrally using this frequency-based test.

McDonald-Kreitman tests also revealed no non-neutral behavior at *Acp53Ea*, *Acp62F*, or *Acp70A*. The only comparisons that showed deviation from neutrality were between *D. p. bogotana* and *D. miranda* in *Acp26Aa* ($p = 0.005$) and between *D. persimilis* and *D. miranda* at *Acp32CD* ($p = 0.0079$). All other comparisons between taxa at *Acp26Aa* and *Acp32CD* did not deviate from neutrality under this test (Table 4.4). These results remained significant after applying the Williams' correction for independence (Sokal and Rohlf 1995).

In *D. pseudoobscura*, *D. persimilis*, and *D. miranda*, *Acp26Aa* contained more replacement polymorphisms than synonymous polymorphisms (Table 4.3). Additionally, more replacement polymorphisms were observed for *Acp62F* in *D. persimilis* and *D. miranda*, while *D. pseudoobscura* and *D. p. bogotana* have nearly equivalent amounts of synonymous and replacement polymorphisms at this locus. On the other hand, *Acp32CD* had more silent polymorphisms than replacement polymorphisms in *D. pseudoobscura* and *D. persimilis*, while *D. p. bogotana* harbored more replacement polymorphisms and *D. miranda* contained no polymorphisms. *Acp53Ea* had roughly equal numbers of replacement and silent polymorphisms for all taxa and *Acp70A* had more silent polymorphisms than replacement polymorphisms for all taxa evaluated.

Tests for Positive Selection on Nucleotide Substitutions

*d*N/*d*S ratios (ω) averaged across lineages and sites were smaller than one for all Acps in both subgroups, with the exception of *Acp32CD* in the *D. melanogaster* subgroup (Table 4.5). However, these Acps likely contain constrained amino acid sites that mask the signature of positive selection at specific amino acids in the protein. The Bayes empirical Bayes approach of Yang et al. (2005) identified many specific residues

subject to positive selection in three of the Acps examined: *Acp26Aa*, *Acp32CD*, and *Acp62F* (Table 4.5). For Acp26Aa, a higher proportion of sites underwent positive selection in *D. pseudoobscura* group than in the *D. melanogaster* group. A similar number of sites underwent positive selection between the groups for *Acp62F*. *Acp53Ea* also had a $\omega > 1$ in the *D. melanogaster* subgroup, but this was not significant (Table 4.5).

Acp26Aa was under the heaviest positive selection in both the *D. pseudoobscura* and the *D. melanogaster* subgroups. *Acp62F* was also undergoing significant positive selection in both groups, but at fewer sites and with lower ω values. No significant positive selection was detected in *Acp53Ea* or *Acp70A* for either group. Acp32CD was undergoing significant positive selection in the *D. melanogaster* group, but not in the *D. pseudoobscura* group, although positive selection was suggested at more sites in this Acp than in either *Acp53Ea* or *Acp70A*. The extensive divergence between orthologous loci in the two clades excluded us from determining whether the same sites were under selection in the two radiations.

Indel Substitutions

Nucleotide substitutions were not the only source of variation in Acp26Aa. Amino acid alignments of Acp26Aa revealed several indels in both the *D. pseudoobscura* and *D. melanogaster* subgroups, including polymorphisms within species for both groups (Figure 6a). In contrast to these exonic indels, there were no indels present in an immediately adjacent 68 bp intron of *Acp26Aa* (data not shown). In the *D. pseudoobscura* group, positively selected sites (with posterior probabilities over 0.8) fell within the insertion / deletion sections of Acp26Aa (Fig. 4.6a). In the *D. melanogaster* group, however, most positively selected sites fell outside of indel regions (Figure 4.6b).

Acp32CD also contained several indels. Although Acp32CD showed no significant positive selection over all sites of the protein, three of the ten sites identified as undergoing positive selection fell within indels in the *D. pseudoobscura* subgroup (data not shown). In addition, there is a single indel polymorphism within Acp32CD of *D. pseudoobscura*. Alignments of Acp32CD revealed one six base pair insertion/ deletion between *D. melanogaster* (USA and Zimbabwe) and *D. simulans*. No indels were present in Acp53Ea, Acp62F, or Acp70A in either of these groups.

DISCUSSION

We have demonstrated that the accessory gland proteins *Acp26Aa* and *Acp62F* have sites that are undergoing positive selection in the *D. pseudoobscura* subgroup. The *D. melanogaster* subgroup has similar proportions of positively selected sites these same two Acps, as well as in *Acp32CD*. Two additional Acps, *Acp53Ea* and *Acp70A*, were not subject to positive selection in either of these subgroups. In addition to this positive selection acting on nucleotide substitutions, we also found several indel replacements and polymorphisms in Acp26Aa and Acp32CD. The regions where these indels occur are the same places that harbor positively selected nucleotide substitutions for Acp26Aa in the *D. pseudoobscura* group, but not in the *D. melanogaster* group. The deep divergence in Acps from the two subgroups prevented us from determining whether the same residues are subject to positive selection in both subgroups. *Acp26Aa* has already been demonstrated to undergo positive selection in the *D. melanogaster* group (Tsaur and Wu 1997; Tsaur *et al.* 1998; Begun *et al.* 2001) and in the *D. pseudoobscura* group (Wagstaff and Begun 2005). However, this is the first study to document positive selection at particular sites for Acp26Aa or any other drosophilid Acp.

Table 4.3. Polymorphism statistics for each Acp in the *D. pseudoobscura* subgroup

Locus	Species	n[a]	L[b]	S[c]	syn[d]	non[d]	θ[e]	π[f]	D[g]	Div[h]
Acp26Aa	*D.pseudoobscura*	20	558	56	24	32	0.03075	0.02733	-0.60919	0.09381
	D. p. bogotana	11	618	16	9	7	0.01255	0.01449	0.86464	0.08313
	D. persimilis	7	654	17	6	11	0.01174	0.01239	-0.05404	0.08622
	D. miranda	3	579	2	0	2	0.00230	0.00173	---	
Acp32CD	*D.pseudoobscura*	20	875	36	25	11	0.01127	0.00834	-1.33668	0.03065
	D. p. bogotana	11	900	9	3	6	0.00545	0.00834	-0.82943	0.03465
	D. persimilis	7	879	17	13	4	0.00928	0.00956	0.21615	0.02782
	D. miranda	3	885	0	0	0	0.00000	0.00000	---	
Acp53Ea	*D.pseudoobscura*	20	330	7	4	3	0.00639	0.00359	-1.54707	0.01199
	D. p. bogotana	11	330	2	1	1	0.00234	0.00238	0.06935	0.01162
	D. persimilis	7	330	1	1	0	0.00165	0.00152	-0.61237	0.01086
	D. miranda	3	330	5	3	2	0.01010	0.01010	---	
Acp62F	*D.pseudoobscura*	20	408	27	14	13	0.01924	0.01399	-1.08969	0.03345
	D. p. bogotana	11	408	6	3	3	0.00567	0.00657	0.73429	0.03380
	D. persimilis	7	408	9	2	7	0.01059	0.00980	-0.52640	0.02647
	D. miranda	3	408	7	2	5	0.01144	0.01162	---	
Acp70A	*D.pseudoobscura*	20	165	1	1	0	0.00191	0.00160	-0.34144	0.04329
	D. p. bogotana	11	165	0	0	0	0.00000	0.00000	-----	0.05455
	D. persimilis	7	165	1	1	0	0.00331	0.00404	1.63299	0.04545
	D. miranda	3	165	2	2	0	0.01212	0.01212	----	

n[a]: Number of lines sequenced.
L[b]:Average length (bp) of the sequences from each species.
S[c]: Number of polymorphic sites.
syn[d]: Number of synonymous (syn) polymorphisms in the coding regions.
rep[d]: Number of nonsynonymous (non) polymorphisms in the coding regions.
θ[e]: Estimate of 4Nu per base pair using the number of polymorphic sites (Watterson 1975).
π[f]: Estimate of 4Nu using the average number of nucleotide differences per site (Nei 1987).
D[g]: Tajima's statistic (1989*b*) (No values were significantly different from zero)
Div[h]: Average divergence per base pair between alleles from each taxon and the alleles of *D. miranda*.

Table 4.4. McDonald-Kreitman Tests of Neutral Molecular Evolution at each Acp locus for the *D. pseudoobscura* group

Locus		*D. p. bogotana*					*D. pseudoobscura*					*D. persimilis*				
		Polymorphic		Fixed			Polymorphic		Fixed			Polymorphic		Fixed		
		Syn	Rep	Syn	Rep	p*	Syn	Rep	Syn	Rep	p*	Syn	Rep	Syn	Rep	p*
Acp26Aa																
	pse	22	27	0	0	---										
	per	14	15	0	1	---	24	33	0	0	---					
	mir	9	5	9	31	0.005	20	26	7	28	---	3	9	10	33	0.9000
Acp32CD																
	pse	27	16	0	0	---										
	per	16	10	3	4	0.377	31	14	0	3	---					
	mir	3	6	11	17	0.745	22	10	7	13	0.017	11	4	5	13	0.0079
Acp53Ea																
	pse	5	4	0	0	---										
	per	1	1	0	0	---	5	3	0	0	---					
	mir	4	3	1	0	---	7	5	1	0	---	4	2	1	0	---
Acp62F																
	pse	15	13	0	0	---										
	per	5	9	3	0	---	15	18	0	0	---					
	mir	4	9	3	5	0.752	15	19	0	3	---	4	11	0	4	---
Acp70A																
	pse	1	0	2	0	---										
	per	1	0	2	0	---	2	0	0	0	---					
	mir	2	0	3	5	---	3	0	1	5	---	3	0	1	5	---

*Probability determined by G-test.

Table 4.5. Likelihood ratio test of positive selection for Acps in both *D. pseudoobscura* and *D. melanogaster* subgroups

Gene	Subgroup	N Alleles/ Taxa	*L* (codons)	*S*	*d*N/*d*S	2Δℓ, M8 vs. M7	Parameter estimates under M8 (beta & ω)	Positively selected sites†
Acp26Aa							p0= 0.933	19, 23, *30*, 31, *32*, *33*, **35**, 38, 39, 40, *45*, 54, 55,
	D. pseudoobscura	32 / 4	244	1.201	0.556	16.9**	p1= 0.067	57, 59, *62*, **63**, 64, *74* , 77, *85*, 89, 98, 101, *117*,
							ω = 3.835	156, 190, 220, 237
							p0= 0.938	
	D.melanogaster	12 / 5	256	0.849	0.870	13.8**	p1= 0.062	2, **23, 24,** 25, 27, **30,** 35, 36, *69*, *95*, *97*, 101,
							ω = 6.753	178, *184*, 187, 200, 204
Acp32CD							p0= 0.855	
	D. pseudoobscura	31/ 4	303	0.394	0.263	2.24	p1= 0.145	145, *162*, 165, 166, 270, 271, **272,** 292, 302
							ω = 1.641	
							p0= 0.892	
	D.melanogaster	8 / 2	260	0.086	1.580	13.2**	p1= 0.108	*22*, 29, 40, *101*, *109*, 145, *184*,
							ω = 17.589	185, 187, *191*, *241*, *250*, *252*
Acp53Ea								
	D. pseudoobscura	31 / 4	110	0.130	0.182	0.001	NA	None detected
							p0= 0.767	
	D.melanogaster	19 / 5	110	0.463	0.266	6.68	p1= 0.233	9, *19*, 21, 30, 108, 109
							ω = 1.194	
Acp62F							p0= 0.943	
	D. pseudoobscura	34 / 4	135	0.808	0.371	7.92*	p1= 0.057	**13**, **16, 86,** 89, 94, *122,124*, 126, 129, 130 131,
							ω = 4.112	132, *133*, **134**
	D.melanogaster	17 / 5	92	1.133	0.450	6.26*	p0= 0.721	7, **9**, 10, 13, 15, 22, 23, 24, 30, 34, **38,** 44, **58**, 70,
							p1= 0.279	81, 85, *90*, **91**
							ω = 1.802	
Acp70A								
	D. pseudoobscura	21 / 4	55	0.237	0.266	0.006	NA	None detected
							p0= 1.000	
	D.melanogaster	7 / 4	55	0.330	0.365	0.002	p1= 0.000	19
							ω = 1.000	

N is reported as number of alleles / number of taxa; *L* is the number of codons, *S* is the tree length, measured as the number of nucleotide substitutions per codon, and *d*N/*d*S is the average ratio over sites and branches, both calculated under model M0. * indicates significance at 5% level; ** indicates significance at 1% level. The proportion of sites under positive selection (*p*1) or under selective constraint (*p*0) are given under model M8. Positively selected sites with posterior probability >0.9 are underlined, 0.8–0.9 in bold, 0.7–0.8 in italics, and 0.5– 0.7 in plain text. † Positively selected sites are identified under Bayes Empirical Bayes (BEB) analysis and are subgroup specific because comparisons of positively selected sites could not be made between subgroups. Sites identified as positively selected are specific to the subgroup they are listed for (i.e. Site 23 of *Acp26Aa* in the *D. pseudoobscura* group is not the same as site 23 in the *D. melanogaster* group)

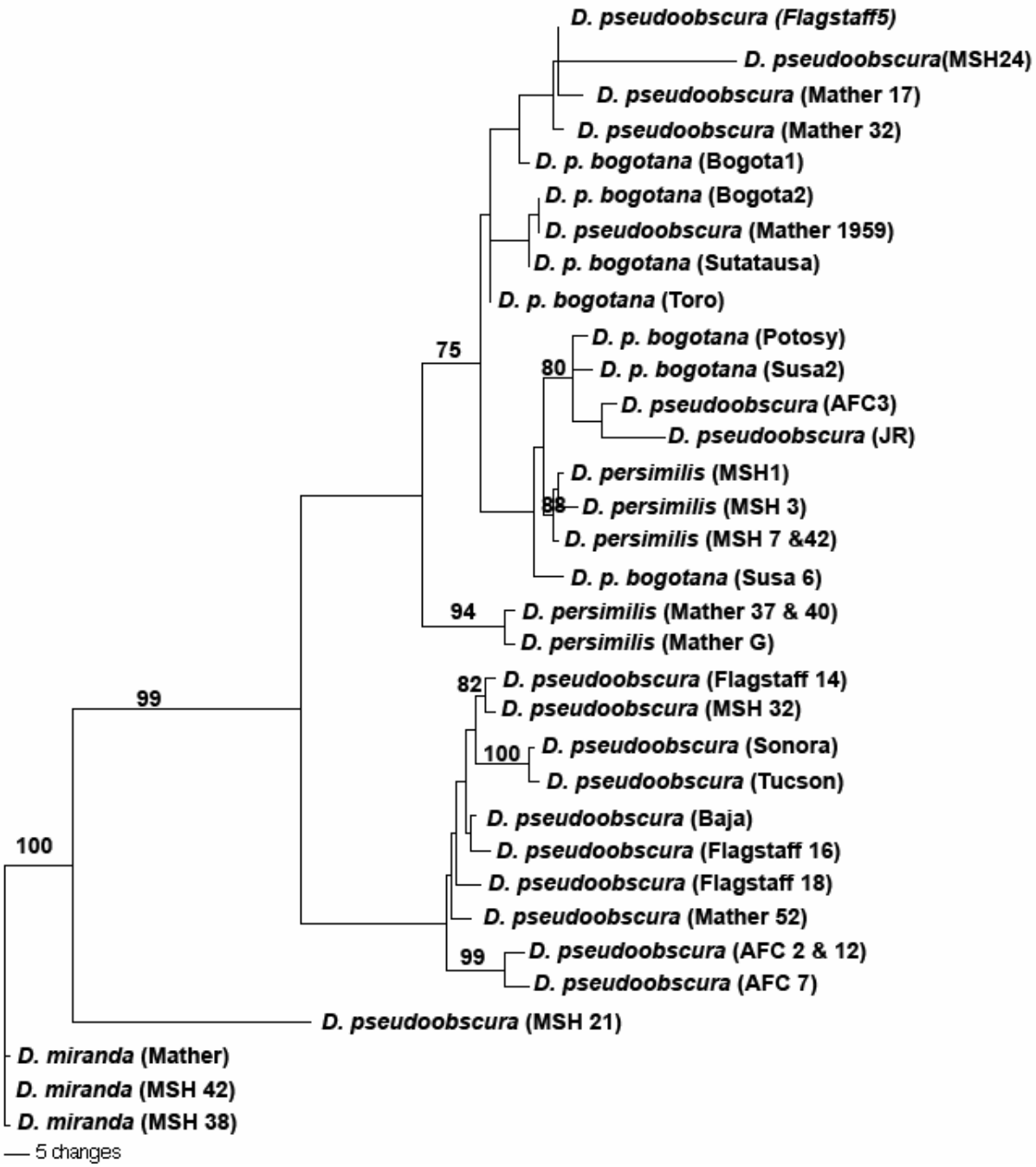

Figure 4.1. Neighbor joining tree for alleles of Acp26Aa

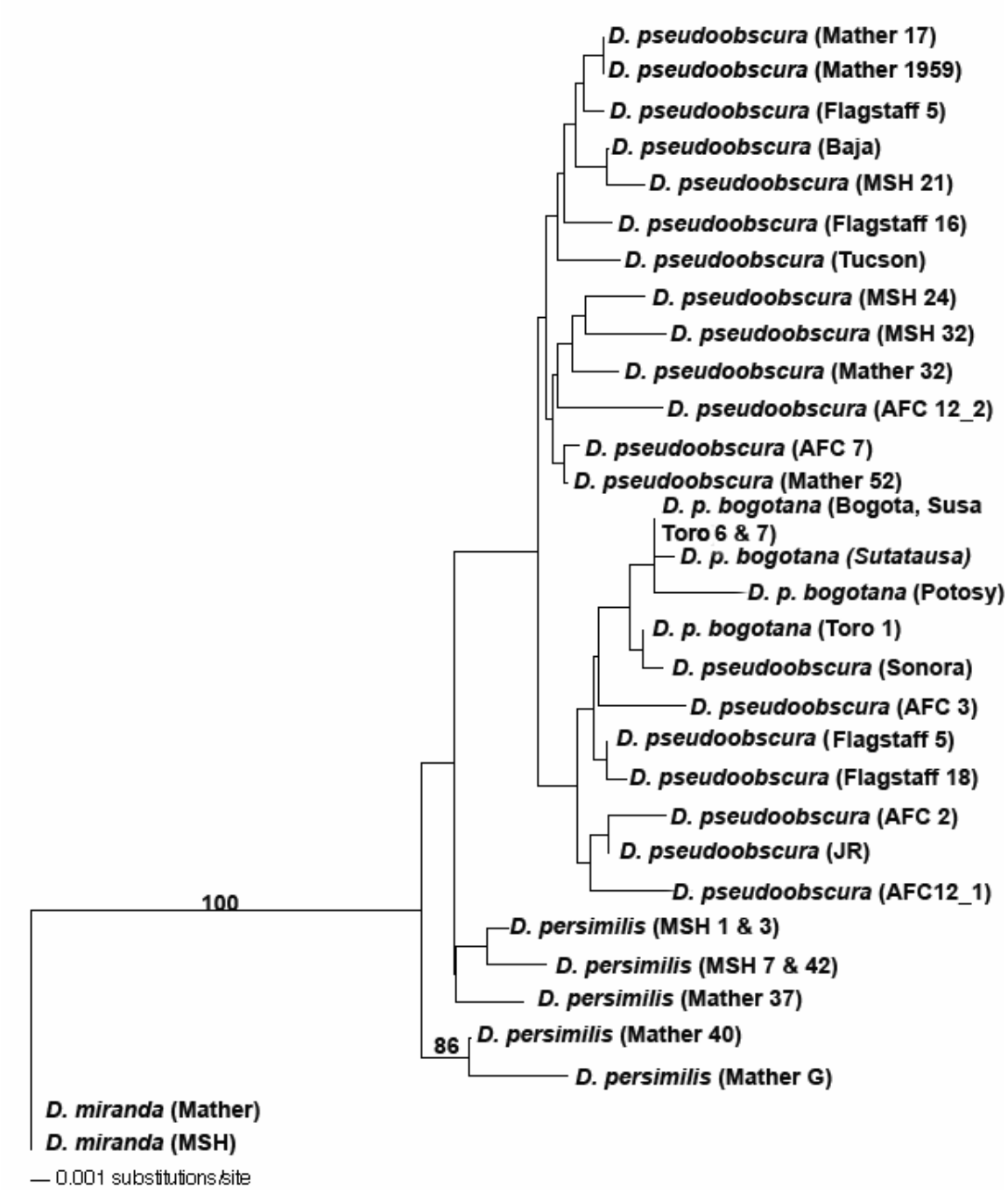

Figure 4.2. Neighbor joining tree for alleles of Acp32CD

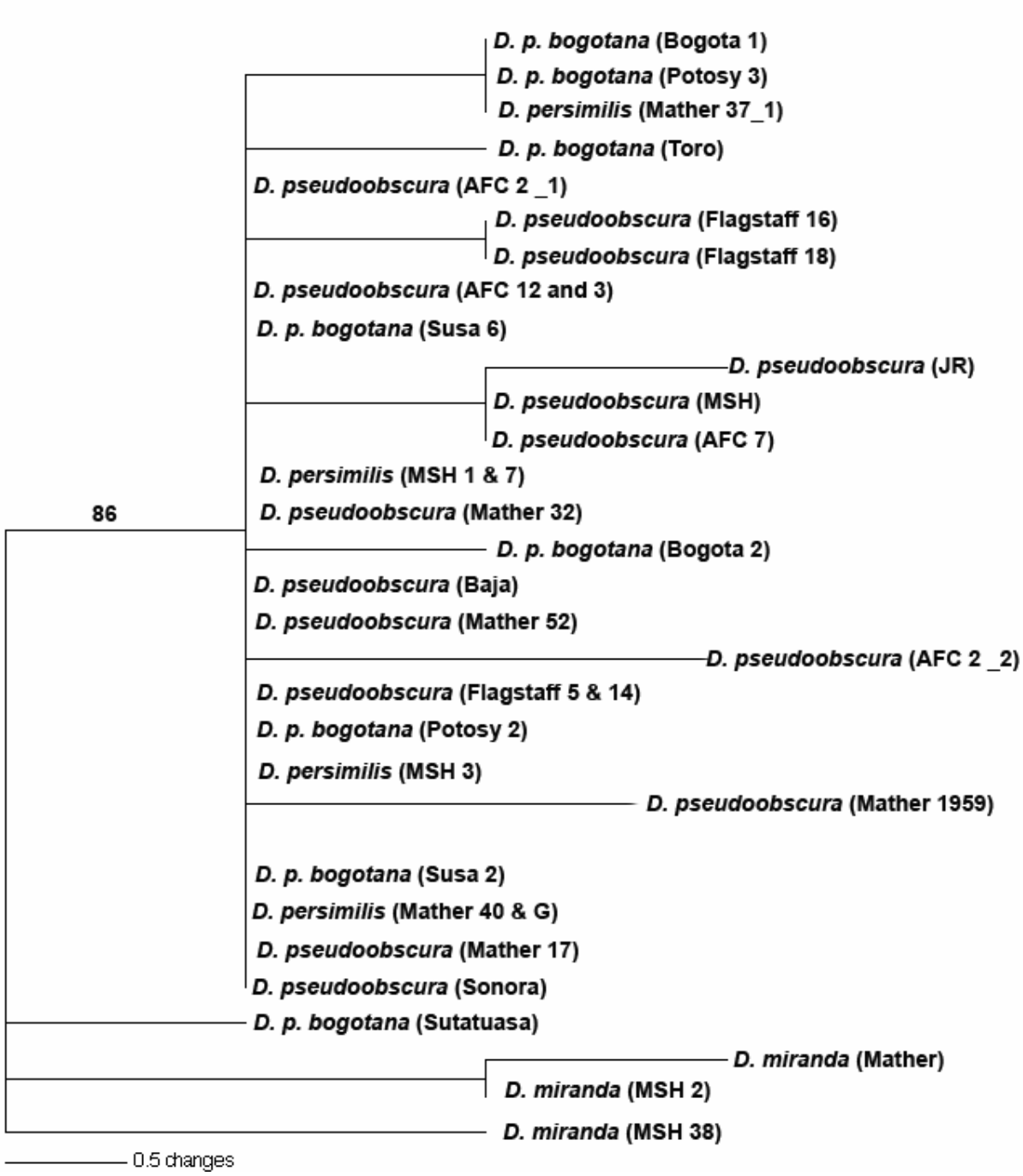

Figure 4.3. Neighbor joining tree for alleles of Acp53Ea

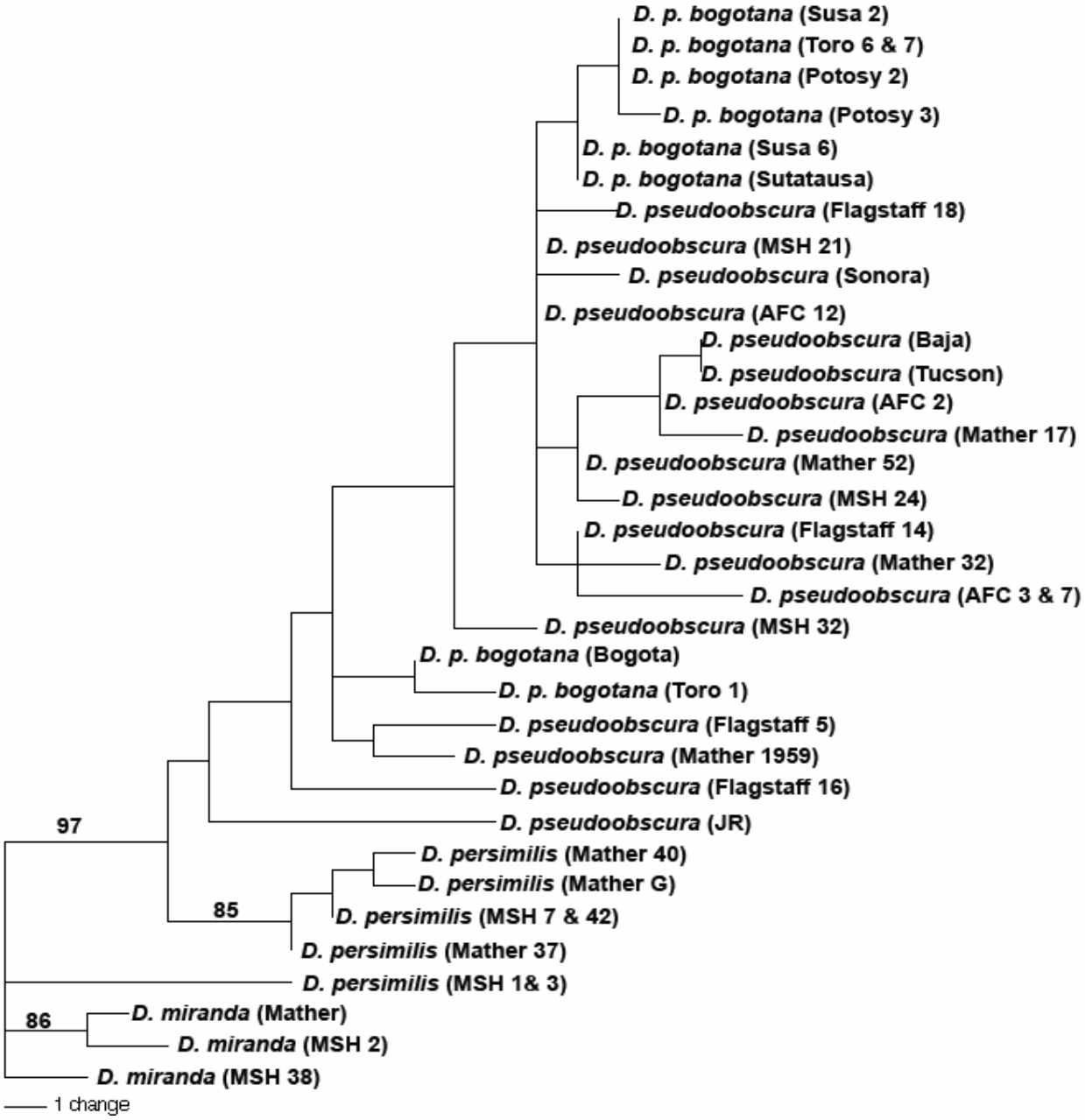

Figure 4.4. Neighbor joining tree for alleles of 62F

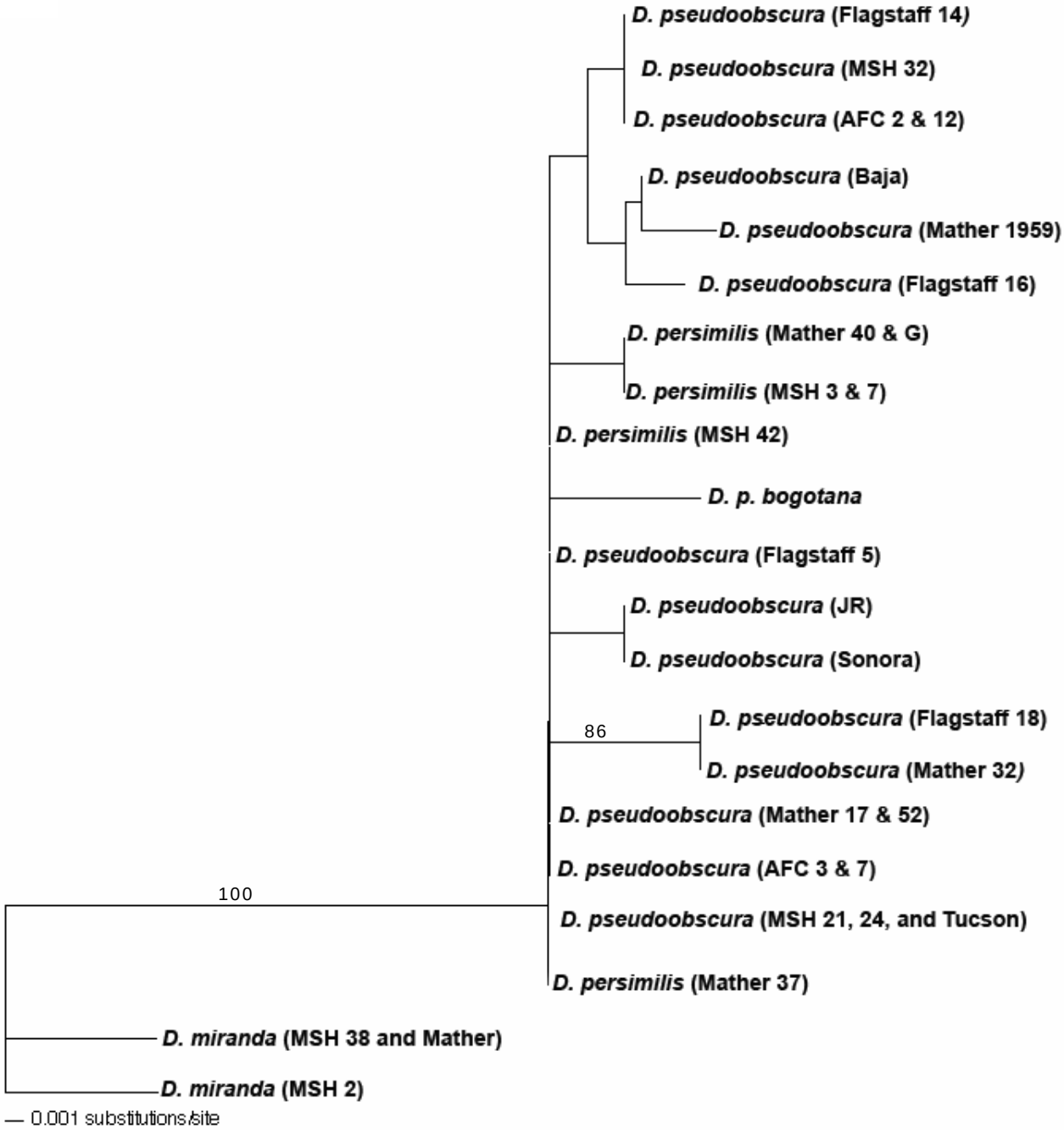

Figure 4.5. Neighbor joining tree for alleles of 70A

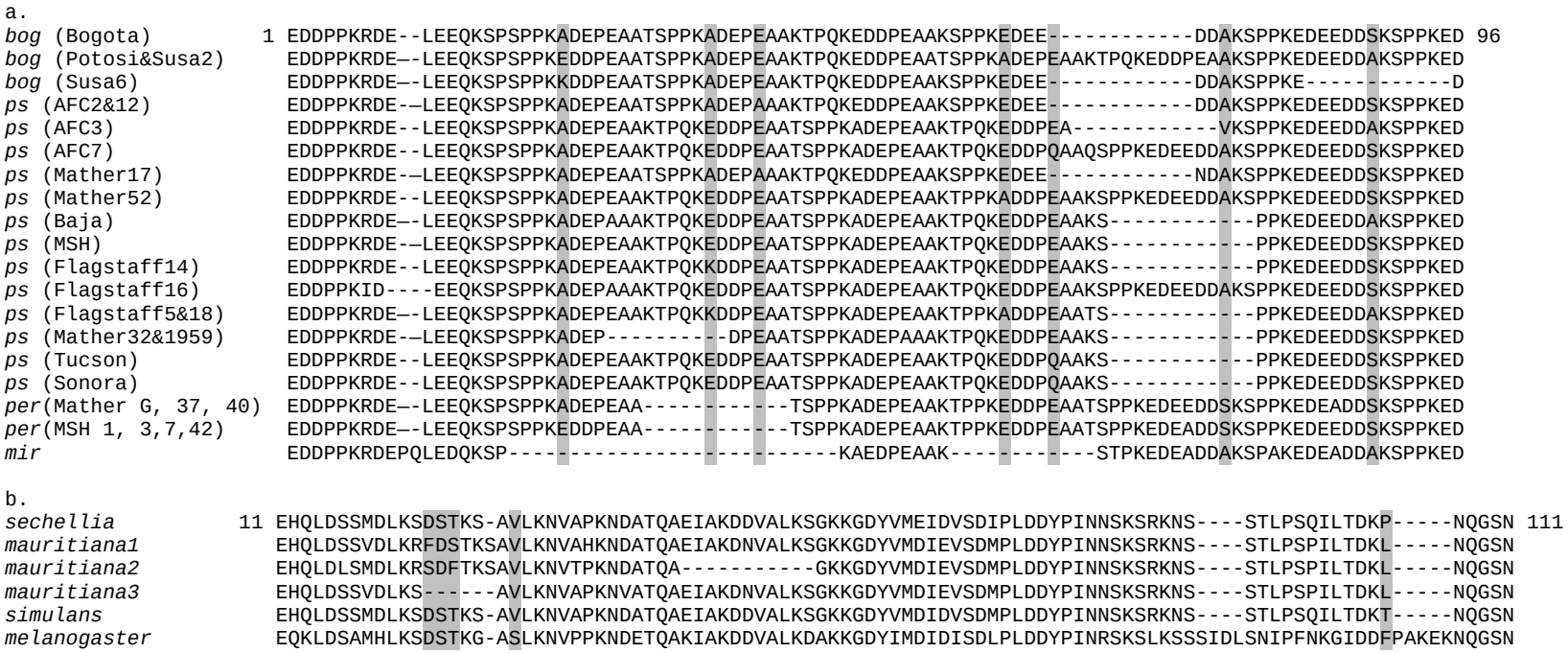

Figure 4.6. Amino Acid alignment of insertion/deletion segment of Acp26Aa

a. *D. pseudoobscura* subgroup amino acid alignment of an insertion/deletion segment of Acp26Aa.

b. *D. melanogaster* subgroup amino acid alignment of an insertion/deletion segment of Acp26Aa.

Positively selected sites with posterior probabilities > 0.8 are highlighted in gray.

Table 4.6. Estimated indel substitution rates for Acp26Aa and, intronic, and gene flanking regions

Region	Taxa 1	Taxa2	Divergence	Indels	Total base pairs	Indel substitution rate (substitutions / year)
Acp26Aa	*D. p. bogotana*	*D. miranda*	2.1 MY	5	732	1.626×10^{-9}
Acp26Aa	*D. pseudoobscura*	*D. persimilis*	0.5 MY	7	732	9.563×10^{-9}
Acp26Aa	*D. pseudoobscura*	*D. p. bogotana*	0.15 MY	6	732	2.732×10^{-8}
intronic†	*D. simulans*	*D. sechellia*	2.3 MY	44	6302	1.520×10^{-9}
5' intergenic†	*D. simulans*	*D. sechellia*	2.3 MY	9	3094	6.324×10^{-10}
3' intergenic†	*D. simulans*	*D. sechellia*	2.3 MY	18	3159	1.290×10^{-9}

† Data from Halligan *et al* 2004

The relative strength of positive selection on nucleotide substitutions acting on the five Acps we examined appears to be the same in two lineages we studies, despite the fact that they split 21-46 million years ago (Beckenbach *et al.*1993). This suggests that the presumably conserved functions of these proteins remain targets of diversifying selection over long periods of time. In contrast, selection for sperm size varies greatly across the cactophilic *Drosophila* of the *D. repleta* group. Within this group, sperm length can be extreme and can extend over 58 mm for the giant sperm species *D. bifurca* (~20X the size of the fly itself) (Pitnick *et al.* 1995). In addition, there is selection for intraspecific variation for sperm size in another member of this group, *D. mojavensis* (Pitnick *et al.* 2003), suggesting that selection acts differently on the various aspects of Drosophila mating.

The functions of the two Acps shown here to be under positive selection suggest a potential role in some observed reproductive incompatibilities within the two subgroups. *Acp26Aa* has been shown to increase egg-laying (Herndon and Wolfner 1995; Heifetz *et al.* 2000; Chapman *et al.* 2001; Heifetz *et al.* 2001) and to be involved in sperm competition (Clark *et al.* 1995) in *D. melanogaster*. In addition, Clark *et al.* (1995) showed correlations between *Acp26Aa* genotypes and sperm displacement ability in *D. melanogaster*. *Acp26Aa* thus *may* play a role in the conspecific sperm precedence observed between *D. pseudoobscura* and *D. p. bogotana* (Dixon *et al.* 2003). Here, the authors used *D. pseudoobscura* populations from Flagstaff and AFC 12 and *D. p. bogotana* populations from Sutatausa and Susa6. Interestingly, Acp26Aa alleles from the *D. pseudoobscura* and *D. p. bogotana* populations fell into different (although weakly supported) phylogenetic groups (Figure 4.1), indicating that there is divergence between

the subspecies in *Acp26Aa*. In addition, *Acp62F* protects sperm from proteolysis (Lung *et al.* 2002) which would protect the sperm in the female's reproductive tract. However, it is unknown if the action of *Acp62F* is species-specific in the *D. melanogaster* subgroup. The rapid evolution of these two proteins may be responsible for some of the postmating / prezygotic reproductive isolation seen in the *D. pseudoobscura* subgroup (e.g., Dixon *et al.* 2003).

Two other Acps that have been shown to be positively selected between *D. melanogaster* and *D. simulans* are Acp36DE and Acp29Ab (Aguadé 1999, Begun *et al.* 2000). However, these genes could not be identified in *D. pseudoobscura* (Wagstaff and Begun 2005) and therefore were not evaluated in this study. Furthermore, Clark *et al.* (1995) demonstrated that there are associations between Acp36DE and Acp29Ab and the sperm's ability to compete in the female's reproductive tract in the *D. melanogaster* group.

Previous studies that evaluate positive selection acting on Acps have only examined positive selection acting on nucleotide substitutions. However, nucleotide substitutions may not be the only target of positive selection. Two recent studies have shown positive selection acting on indels in a sperm-specific protein (Catsper1) in both primates (Podlaha and Zhang 2003) and rodents (Podlaha *et al.* 2005). Indels in Catsper1 may effect sperm motility and thus this protein may help mediate sperm competition (Podlaha *et al.* 2003, Podlaha *et al.* 2005). Positive selection has also shown to occur in indel rich regions of nucleotide binding site (NBS)-LRR gene family of *Arabidopsis thaliana* (Mondragón-Palomino *et al.* 2000), and in the gamete recognition protein bindin from sea urchins (Metz and Palumbi 1996; McCartney and Lessios 2004). Previous studies

evaluating the molecular evolution of Acps in *Drosophila*, however, have either implicitly or explicitly excluded indels from their analyses (e.g., Tsaur and Wu 1997; Begun *et al.* 2000). Our results suggest that indel substitutions play a significant role in the divergence of some Acps.

Determining whether indel substitutions are promoted by positive selection is not as straight forward as for nucleotide substitutions. To test whether the rate of indel substitutions in Acps are significantly higher than the neutral expectation, the indel substitution rate of potential selected indel substitutions can be compared to those in neutral (non-coding) sequences. A conservative indel substitution rate can be calculated as described in Podlaha and Zang (2003): (Number of nucleotide indels)/ (total number of base pairs)/ (divergence date x 2). For example, in Acp26Aa, there were 5 indels out of the total 732 base pairs sequenced between *D. miranda* and *D. p. bogotana*, which diverged 2.1 MYA. The indel substitution rate at this locus can thus be estimated as:

$$(5) / (732) / ((2.1 * 10^{6}) * 2) = 1.626 \times 10^{-9} \text{ indel substitutions per year.}$$

We also performed the same calculation for indels in intronic, 5' intergenic, and 3' intergenic regions between *D. simulans* and *D. sechellia* (Halligan *et al* 2004). The indel substitution rates in Acp26Aa is higher than, or of the same order of magnitude as, noncoding regions of *Drosophila* genomes (Table 4.6). Note that this method of comparison is conservative because indels occurring in exonic sequences (as in Acp26Aa) must occur in multiples of three bps so as not to disrupt open reading frames, a constraint not present for non-coding regions.

Positive selection often drives the rapid evolution of reproductive proteins (Swanson and Vacquier 2002). We have demonstrated that the strength of positive

selection on nucleotide substitutions acting on five Acps is relatively the same in two drosophilid lineages that split 21-46 million years ago (Beckenbach *et al.*1993). In addition, positive selection on indels may also contribute heavily to the divergence of some Acps, and may even be promoted by positive selection. Further studies are needed to determine the physiological functions and fitness consequences of these Acps in the *D. pseudoobscura* group to elucidate their possible roles in the evolution of reproductive isolation.

CHAPTER FIVE:

CONCLUSIONS

If reproductive barriers have not evolved completely when two allopatric populations come into secondary contact, interbreeding may occur. If these matings produce maladaptive hybrids, there is a cost associated with such matings. Thus, potentially reproducing populations may evolve barriers that prevent their interbreeding. Premating isolation barriers can, by definition, prevent mismatings from occurring. If interspecific mating does occur, subsequent postmating / prezygotic isolating barriers may prevent the formation of an unfit hybrid zygote. Finally, in the event of a successful mismating and the formation of a zygote, postzygotic isolation barriers are present in the form of inviable or infertile offspring.

Although there have been numerous studies on the evolution of postzygotic isolation between taxa (reviewed in Orr *et al.* 2004), our understanding of prezygotic barriers to gene flow lags behind. This dissertation addresses prezygotic reproductive isolation at three levels: reinforced behavioral discrimination between two species; conspecific sperm precedence between two subspecies; and rapid divergence of reproductive proteins within and between entire subgroups. The data presented in this dissertation furthers the understanding of prezygotic reproductive isolation and its role in speciation by 1) establishing that reinforcement can result as a reduction in male courtship behaviors that a female will accept, 2) demonstrating that conspecific sperm precedence can evolve at the subspecies level, suggesting it may be involved in the initial steps toward speciation, and 3) showing that the rapid evolution of reproductive proteins in the *D. melanogaster* group also occurs in another distant linage.

Reinforcement is the process by which natural selection increases premating reproductive isolation to prevent maladaptive hybridization in sympatry (Dobzhansky

1940). Although reinforcement has been studied extensively, including in the *D. pseudoobscura* subgroup (e.g., Noor 1995; Ortíz-Barrientos *et al.* 2004), the manner in which reinforcement evolves remains unknown. My results in chapter two suggest that reinforcement between *D. pseudoobscura* and *D. persimilis* evolved via the discrimination enhancement model. The signature of reproductive character displacement was detected in matings between *D. pseudoobscura* and *D. persimilis: D. persimilis* females discriminated equally between *D. pseudoobscura* males from allopatric and sympatric populations. This is in contrast to the alterative hypothesis (preference evolution), which predicts that *D. persimilis* females would prefer *D. pseudoobscura* males from sympatric populations. This has also been suggested in other taxa. For example, a potential case of reinforcement by male discrimination enhancement between the Galapagos finches *Geospiza difficilis* and *G. fuligznosa* was described by Ratcliffe and Grant (1983). The observation that reinforcement arises via discrimination enhancement may help to determine the likelihood of speciation by reinforcement in other taxa that exhibit discrimination enhancement.

In spite of premating barriers, matings between species that may produce maladaptive hybrids still commonly occur in the wild. Nonetheless, postmating / prezygotic barriers are often present to prevent to gene exchange. One such barrier is conspecific sperm precedence; if a female is mated to both conspecific and heterospecific males, she will preferentially produce conspecific rather than hybrid offspring (Howard 1998). The results presented in chapter three suggest that conspecific sperm precedence may have evolved in the early stages of divergence between *D. pseudoobscura* and *D. p. bogotana*. However, these subspecies already possess other barriers to genetic exchange

(weak mating discrimination and one way hybrid sterility); therefore which barrier to gene exchange evolved first is not known. Prior to this work, conspecific sperm precedence had only been demonstrated between taxa that were already considered to be good species. The work presented in chapter three demonstrates that conspecific sperm precedence at the subspecies level for the first time and shows that it may act between taxa that are undergoing the early stages of speciation.

Conspecific sperm precedence could not be evaluated in *D. pseudoobscura* and *D. persimilis* due to reinforcement. Mating discrimination between these two taxa is too strong to obtain the first and second matings required to evaluate conspecific sperm precedence. However, one can speculate that conspecific sperm precedence would be observed between *D. pseudoobscura* and *D. persimilis,* as it is between *D. simulans* females and the males of *D. sechellia* or *D. mauritiana* (Price 1997) because both groups are similar with regard to the amount of reproductive isolation they possess. Both pairings between *D. pseudoobscura* and *D. persimilis* and matings between *D. simulans* and *D. sechellia* (or *D. mauritiana*) produce all sterile males and fertile females (David *et al.* 1974; Lemeunier *et al.* 1986). Thus, both groups exhibit a strong barrier to gene exchange.

In addition, the subspecies *D. pseudoobscura* and *D. p. bogotana* also exhibit mating discrimination. Second matings occurred seven days after first matings and these subspecies displayed weak yet significant conspecific sperm precedence. In experiments demonstrating stronger conspecific sperm precedence between *D. simulans* females and *D. sechellia* or *D. mauritiana* males (Price 1997), second matings occurred two days after first matings. If we had been able to get *D. pseudoobscura* and *D. p. bogotana* to remate

in less than seven days, perhaps the conspecific sperm precedence observed between the subspecies would be comparable to that seen between other species of *Drosophila*.

All of the processes contributing to prezygotic reproductive isolation are governed by species-specific proteins (e.g., pheromones, seminal fluid proteins, etc). The accessory gland proteins (Acps) of *Drosophila* elicit many changes in mated females, including reducing their willingness to remate. Many of the proteins involved in reproduction are undergoing accelerated rates of evolution (see Swanson and Vacquier 2002). The results in chapter four demonstrate that *Acp26Aa* and *Acp62F* both contain sites that are undergoing positive selection in the *D. pseudoobscura* subgroup. Furthermore, the *D. melanogaster* subgroup has similar proportions of positively selected sites these same two Acps. In *D. melanogaster*, Acps facilitate many behavioral and physiological changes in the mated females. Specifically, *Acp26Aa* increases egg-laying (Herndon and Wolfner 1995; Heifetz *et al.* 2000; Chapman *et al.* 2001; Heifetz *et al.* 2001) and is involved in sperm competition (Clark *et al.* 1995) and *Acp62F* protects sperm from proteolysis (Lung *et al.* 2002). Although both of these functions have only been elucidated in *D. melanogaster*, they have been identified as orthologous proteins in *D. pseudoobscura* (Wagstaff and Begun 2005) and thus may serve similar functions.

Protein divergence is not brought about exclusively by nucleotide substitutions. Many reproductive proteins harbor insertion/ deletion mutations in addition to nucleotide substitutions. Recent studies (Podlaha and Zhang 2003; Podlaha *et al.* 2005), including the data from chapter four, also support the idea that indel substitutions are positively selected for in the evolution of reproductive proteins. Thus, indels may play a key, but

previously ignored, role in the rapid evolution of reproductive proteins, including *Drosophila* Acps.

This dissertation contributes to the elucidation of the role of prezygotic reproductive isolation in the process of speciation by studying three types of prezygotic reproductive isolation in the *Drosophila pseudoobscura* subgroup. Species in this subgroup have evolved at least three significant prezygotic barriers to gene exchange, in addition to the postzygotic barriers they already harbor. The strong mating discrimination and reinforcement exhibited between *D. pseudoobscura* and *D. persimilis,* and the mating discrimination between *D. pseudoobscura* and *D. p. bogotana*, prevent mismatings that would result in maladaptive hybrids. If mismatings do occur, conspecific sperm precedence and rapid accessory gland protein evolution increase the odds that only non-hybrid offspring are formed. Prior to the work generated by this dissertation, prezygotic reproductive isolation was only evaluated as reinforcement in the *D. pseudoobscura* subgroup (e.g., Noor 1995; Ortíz-Barrientos *et al* 2004). Together, the chapters of this dissertation provide relevant data toward understanding three forms of prezygotic reproductive isolation, including reinforcement, and there roles in speciation.

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VITA

Sheri Dixon Schully was born on August 16, 1979, in Baton Rouge, Louisiana to Marvin Dale and Lydia Mayeux Dixon. Sheri grew up in Gonzales, Louisiana and attended Galvez Elementary School, Lake Primary School, and Galvez Middle School. She graduated from St. Amant High School with honors in 1997. Sheri began her undergraduate career at Louisiana State University in the fall of 1997. In the spring of 2001, Sheri graduated with her Bachelor of Science from Louisiana State University with a major in zoology and a minor in chemistry. As an exceptional applicant, Sheri was accepted into the Graduate Program of the Department of Biological Sciences at Louisiana State University and began her research in the fall of 2001. Sheri will graduate with a doctorate in biological sciences on August 11, 2005.